EXHIBIT D

RESEARCH/

BY-PASSING IMMUNIZATION: BUILDING HIGH AFFINITY HUMAN ANTIBODIES BY CHAIN SHUFFLING

James D. Marks[1], Andrew D. Griffiths[1], Magnus Malmqvist[1], Tim P. Clackson[1], Jacqueline M. Bye[1,2] and Greg Winter[1,3,*]

[1]MRC Centre for Protein Engineering, MRC Centre, Cambridge CB2 2QH, U.K. [2]MRC Immunopathology Unit, Department of Immunology, AFRC Institute of Animal Physiology and Genetics, Babraham, Cambridge CB2 4AT, U.K. [3]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K. *Corresponding author.

Diverse antibody libraries can be displayed on the surface of filamentous bacteriophage, and selected by panning of the phage with antigen. This allows human antibodies to be made directly *in vitro* without prior immunization, thus mimicking the primary immune response[1]. Here we have improved the affinity of one such "primary" antibody by sequentially replacing the heavy and light chain variable (V) region genes with repertoires of V-genes (chain shuffling)[2] obtained from unimmunized donors. For a human phage antibody for the hapten 2-phenyloxazol-5-one (phOx) ($K_d = 3.2 \times 10^{-7}$ M), we shuffled the light chains and isolated an antibody with a 20 fold improved affinity. By shuffling the first two hypervariable loops of the heavy chain, we isolated an antibody with a further 15-fold improved affinity. The reshuffled antibody differed in five of the six hypervariable loops from the original antibody and the affinity for phOx ($K_d = 1.1 \times 10^{-9}$ M) was comparable to that of mouse hybridomas from the tertiary immune response. Reshuffling offers an alternative to random point mutation for affinity maturation of human antibodies *in vitro*.

For serotherapy, monoclonal antibodies would ideally be of human origin, but human hybridomas are difficult to make and require human immunization (see ref. 3 for review). New technologies have prompted new solutions. For example, gene technology has prompted the 'humanizing' of rodent antibodies by transplanting their hypervariable loops into a human antibody[4-8], leading to clinical application[9]. The use of the polymerase chain reaction[10] (PCR), to clone and express antibody V-genes[11,12] and phage display technology[13,14] to select antibody genes encoding fragments with binding activities[15] has resulted in the isolation of antibody fragments from repertoires of PCR amplified V-genes using immunized mice or humans[2,16] thus by-passing conventional hybridoma technology.

Recently, we reported the isolation of human antibody fragments directed against both small (hapten) and large (protein) antigens from the same single chain Fv (scFv)[17,18] library (3×10^7 members) made from the V-genes of unimmunized healthy blood donors and displayed on the surface of bacteriophage[1]. The process by-passes immunization by mimicking immune selection. Indeed, the antibody fragments were highly specific and had affinities typical of a primary immune response ($K_d = 1 - 5 \times 10^{-7}$ M). The technology appears to have the potential to make human antibodies entirely *in vitro*, but for most practical applications the antibodies need higher affinities typical of later immune responses.

Affinity maturation can be mimicked *in vitro* by making point mutations in the V-genes, for example by using an error-prone polymerase, and selecting mutants for improved affinity[19]. Alternatively, new combinations of antibody heavy and light chains can be made by recombining a single heavy or light chain with a library of partner chains (chain shuffling). Chain shuffling has been used to make new combinations of heavy and light chains with hapten binding activities from the V-genes of immunized animals but affinities of the shuffled antibodies were not measured[2,20]. An attempt to derive hapten binding antibodies by reshuffling the V-genes from an immunized source with those from a naive source failed, prompting the authors to assert that "redesign of antibodies through recombination of a somatically mutated chain with a naive partner may be a difficult process"[20].

For this work, we started with the human antibody (αphOx-15) directed against the hapten 2-phenyloxazol-5-one (phOx) that had been isolated from a phage display library made from unimmunized human donors[1]. Both heavy and light chains of α-phOx-15 are somatically mutated. Using repertoires of heavy and light chain V-genes from unimmunized donors, we reshuffled the heavy chain with the repertoire of light chains, and vice-versa to make shuffled somatically mutated antibodies with higher affinities.

RESULTS

Light chain shuffling. A scFv fragment (αphOx-15) directed against the hapten phOx was isolated from a phage antibody library constructed from the heavy (VH) and light (Vκ and Vλ) chain genes from the peripheral blood lymphocytes of unimmunized human donors[1]. The VH gene of αphOx-15 was assembled with a repertoire of Vκ and Vλ genes from the same unimmunized donors to make shuffled scFv genes[2], and cloned into the phagemid vector pHEN1 (ref. 21) for display as a fusion with gene 3 coat protein[15]. After transformation, the pha-

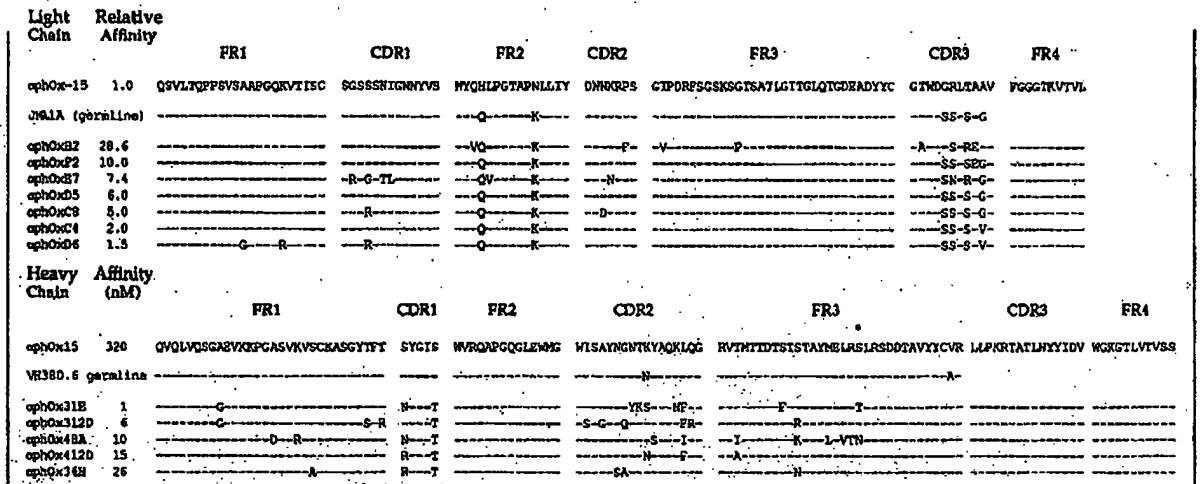

FIGURE 1 Sequences and affinities of the light and heavy chains of phOx binders from the shuffled libraries. The sequences of the light chains are compared to αphOx-15 and the most homologous Vλ germ-line gene JMA1A (ref. 1). The sequences of the heavy chains are compared to αphOx-15 and the most homologous germline gene VH380.6 (ref. 1). Relative affinities were determined by inhibition ELISA and are expressed as I_{50} mutant/I_{50} αphOx15. Affinities were determined by fluorescence quench titration. All antibodies bound phOx specifically (did not bind BSA in an ELISA and binding to phOx-BSA coated microtitre plates could be inhibited by soluble phOx-GABA). *Location of the cloning site for the heavy chain repertoire.

gemid library (2×10^6 clones) appeared diverse by BstNI fingerprinting[2], was rescued with helper phage[1] and subjected to panning on phOx-BSA coated tubes[1]. For expression of soluble scFv, the phage eluted from the tubes were used to infect a non-suppressor strain of bacteria[21] (for details see Experimental Protocol).

To identify clones with improved affinities, the binding of soluble scFvs to phOx-BSA were compared by ELISA. After a single round of panning, soluble scFv from 59/192 clones bound to phOx-BSA with a stronger signal than αphOx-15 scFv whereas before panning, none of 192 clones gave a stronger signal. Six of these clones, and a further 4 clones from a second round of panning, were sequenced. Six unique Vλ light chains were found, all from the same Vλ1-gene family and probably the same germ-line gene as the αphOx-15 light chain (Fig. 1). The human Vλ chains were mutated at a range of sites, diverging by 0 to 9 amino acid residues from the putative Vλ germ-line gene (VλJM1A). The clustering of residue changes, particularly in CDR3, indicates that the mutant light chains were derived directly from the germ-line Vλ-gene rather than the αphOx-15 light chain (Fig. 1).

The scFv fragments from eight different clones were ranked by competition for binding to phOx-BSA with soluble phOx hapten[22], and the "relative affinities" were found to be up to 27 fold higher than αphOx-15 (Fig. 1). The affinities of αphOx-15 and αphOxB2 (the clone with the highest relative affinity) were also measured directly by fluorescence quench titration. The affinity of αphOxB2 was found to be 1.5×10^{-8} M (20 fold higher than αphOx-15) (Table 1). The kinetics of binding (off-rates) of purified αphOx-15 and αphOxB2 scFvs to phOx modified BSA were determined by real-time biospecific interaction analysis based on surface plasmon resonance (SPR, Pharmacia BIAcore)[23,24]. The off-rate was much slower for αphOxB2 but calculated on-rates (k_{on}/K_d) were similar (Table 1). Thus the improved affinity of αphOxB2 is due to its slower off-rate.

Heavy chain shuffling. The reshuffled heavy chain library was prepared as described in the Experimental Protocol. Briefly, a repertoire of VH genes (VH1 family) was amplified by PCR from the IgG and IgM mRNAs of unimmunized donors using primers based in the first and third framework regions. The VH repertoire, which encodes the first two hypervariable loops and three framework regions, was cloned into a vector encoding the third hypervariable loop and the light chain of α-phOxB2. The resulting library (2×10^5 clones) was panned on phOx and soluble scFv screened by ELISA for binding after each round of selection.

TABLE 1 Affinities and kinetics of binding to phOx of original isolate (αphOx15) and chain shuffled mutants.

Clone	Residue changes (from αphOx-15)	K_d*(M)	k_{off}†(s^{-1})	k_{on}§(M^{-1} s^{-1})
Original Isolate				
αphOx-15	0	$3.2 \pm 0.1 \times 10^{-7}$	$4.3 \pm 0.6 \times 10^{-1}$	1.3×10^6
New Light chain				
αphOxB2	10	$1.5 \pm 0.6 \times 10^{-8}$	$1.7 \pm 0.4 \times 10^{-2}$	1.1×10^6
New Light chain and heavy chains				
αphOx34H	16	$2.6 \pm 0.7 \times 10^{-8}$	$7.3 \pm 0.8 \times 10^{-3}$	2.8×10^5
αphOx412D	15	$1.5 \pm 0.4 \times 10^{-8}$	$5.8 \pm 0.6 \times 10^{-3}$	3.9×10^5
αphOx48A	22	$1.0 \pm 0.2 \times 10^{-8}$	$2.5 \pm 0.2 \times 10^{-3}$	2.5×10^5
αphOx312D	20	$6.0 \pm 1.1 \times 10^{-9}$	$3.5 \pm 0.6 \times 10^{-3}$	5.8×10^5
αphOx31E	20	$1.1 \pm 0.4 \times 10^{-9}$	$3.8 \pm 0.5 \times 10^{-3}$	3.5×10^6

*Measured by fluorescence quench titration. †Measured by surface plasmon resonance in BIAcore (Pharmacia).
§Calculated from k_{on}/K_d.

Before selection 0/94 clones bound to phOx whereas after 3 and 4 rounds of selection, 38/94 and 51/94 clones bound to phOx. Supernatants from all 90 clones were screened by SPR for dissociation from phOx-BSA. All 90 clones had slower off-rates than αphOxB2. These clones were grouped according to off-rate and BstNI restriction pattern and eight clones were sequenced (Fig. 1) revealing 5 unique sequences. All 5 were derived from the same germline VH-gene (VH380.6, ref. 1) as αphOx-15 and αphOxB2 but had an additional 5 to 12 residue changes (Fig. 1 and Table 1). Residue 35 was changed from serine to threonine in all 5 mutants.

The affinities of three of the mutants were shown by fluorescence quench titration to be greater than αphOxB2 (Table 1). The affinities ranged from 2.6×10^{-8} M to 1.1×10^{-9} M (12 to 320-fold higher than αphOx-15 and 0.6 to 15-fold higher than αphOxB2). All five mutants had slower off-rates than αphOx-15 or αphOxB2. The highest affinity antibody, αphOx31E, had a faster on-rate than αphOx-15 or αphOxB2.

DISCUSSION

Previously, we used phage display and the V-genes from unimmunized donors to make antibody fragments against both small (hapten) and large (protein) antigens with affinities typical of the primary immune response. While the approach is potentially useful for making therapeutic human antibodies, we need to find ways of increasing the antibody affinities. Here we have shown that this can be accomplished by chain shuffling. We diversified the structure of an antibody by first shuffling light chains, then heavy chains, while retaining the third hypervariable loop of the heavy chain. Much of the sequence and structural variation of antigen binding sites is encoded by this loop, which is located at the center of the antigen binding site[25]. By retaining it, while shuffling the other loops, we aimed to diversify the structure without disrupting the key features of the antigen binding site.

We chose the hapten phOx for our model experiments, as the immune response and affinity and kinetic maturation is well studied[26–28]. The affinities of αphOxB2, from the light chain shuffled library, and the 5 mutants from the heavy chain shuffled library are comparable to that of mouse hybridomas from the secondary or tertiary immune response to the same hapten[26] (Fig. 2). Indeed, of anti-phOx hybridomas from the mouse secondary or tertiary response, only 2 of 24 had a higher affinity than αphOx31E (ref. 26).

The improvement in affinity results almost exclusively from a slower off-rate. Somatic hypermutation of the V-genes used in the murine primary immune response to phOx also improves affinity mainly by slowing the off-rate[26]. The results suggest that our washing and binding conditions favor the selection of phages with slower off-rates rather than faster on-rates in contrast to the suggestion of Garrard et. al.[29]. As we build antibodies with higher and higher affinities, it becomes increasingly likely that the best binders will remain attached to the solid phase, necessitating more vigorous elution conditions.

In vivo, affinity maturation occurs by random mutation of the original heavy and light chain pairings and by the appearance of new heavy and/or light chain pairings (repertoire shift)[27,28]. We can simultaneously mimic aspects of both processes *in vitro* by tapping the natural pool of diverse unmutated and mutated heavy and light chains via chain shuffling. Using V-genes derived from an immunized mouse, we had previously shown that new partners could arise from different V-gene families[2]. In

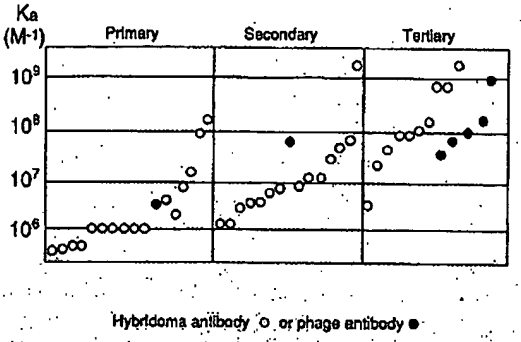

FIGURE 2 Comparison of affinities of anti phOx antibodies from hybridomas and from phage antibodies. Affinity constants (Ka) for anti-phOx hybridomas from primary, secondary and tertiary responses from immunized mice (data taken from ref. 26) are compared with data (Table 1) for phage antibodies from naive phage library (primary), light chain shuffled (secondary) and heavy chain shuffled (tertiary) libraries.

the present study, both the light chains and heavy chains are derived from the same germline gene and the antibodies differ only by point mutations. Nevertheless the repertoire of mutants should differ from those generated by random mutation of the αphOx-15 antibody in two respects. Firstly, the V-genes encoding the shuffled chains have been selected from the mRNA of B-lymphocytes and are more likely to be functional. In contrast, *in vitro* random mutagenesis, for example using an error prone polymerase[19], is likely to result in many mutants that would compromise chain folding, particularly if multiple mutations were introduced into the same gene. Secondly, with *in vitro* mutagenesis mutations are introduced directly into αphOx-15 whereas with chain swapping, mutations are introduced into the corresponding germline genes. This could allow any deleterious mutations in αphOx-15 to be replaced more readily.

A shuffling strategy may be applicable to protein antigens as well as haptens. Although there are a larger number of contacts between protein and antibody, and the chances of disrupting multiple favorable contacts by shuffling is greater, this may be compensated by the loss of multiple unfavorable contacts.

One advantage of building an artificial immune system is that by allowing heavy chains to sample other light chains, and vice-versa we employ a strategy that is not open to the immune system. Thus shuffling enlarges the repertoire size, enhancing the chances of finding higher affinity antibodies[30], and in principle allowing chains with deleterious mutations[31] to be replaced by others. Shuffling chains and hypervariable loops appears to be a powerful way of diversifying antibody structure, and the pool of rearranged V-genes from unimmunized donors provides a rich source of genetic diversity.

EXPERIMENTAL PROTOCOL

Construction of a reshuffled light chain library. A scFv library was assembled[2] from the VH gene of αphOx-15 and a Vλ and Vκ repertoire[1] using PCR. To avoid contamination with the original light chain, the VH gene of αphOx-15 was subcloned into the vector pJM-1 (ref. 2), amplified by PCR using primers HuVH1aBACK and HuJH6FOR[1], purified on a 2% (w/v) agarose gel and isolated from the gel using Geneclean (Bio-101). Reshuffled scFv repertoires were PCR assembled[1] from the phOx-15VH DNA, linker DNA and the same human Vλ and Vκ gene repertoires used to construct the primary library from

which αphOx-15 was isolated[1]. The repertoires were digested with NcoI and NotI, purified on a 1.5% (w/v) agarose gel, electroeluted[32], precipitated with ethanol and ligated into the vector pHEN-1 (ref. 21) digested with NcoI and NotI. The ligation mix was used to transform electrocompetent[33] *E. coli* TG1 (ref. 34). Cells were grown for 1 hour in 1 ml of SOC[32] and then plated on TYE[35] medium with 100 μg/ml ampicillin 1% (w/v) glucose. Colonies were scraped off the plates into 5 ml of 2 × TY[35] broth containing 100 μg/ml ampicillin, 1% (w/v) glucose and 15% glycerol.

Construction of a reshuffled heavy chain library. A scFv library was prepared containing the VH CDR3 and Vλ of αphOxB2 and a repertoire of human VH1 genes. To eliminate potential contamination with the original heavy chain, the human VH1 pseudogene DP-22 (ref. 36) was amplified using PCR from an M13 template using the primers HuVH1BACK-SFI (ref. 1) and HuVH1FR3FOR (5'-GGC CGT GTC ATA TCT CAG-3'), digested with NcoI and BglII, gel purified and ligated into the vector pHEN-1phOxB2 digested with NcoI and BglII. The resulting vector, pHEN-1-ΨVHB2, contained the DP-22 VH1 pseudogene and the VH CDR3 and Vλ of αphOxB2. To prepare a repertoire of human VH1 genes, human PBL RNA was primed in separate reactions with HuIgG1-4CH1FOR and HuIgMFOR and 1st strand cDNA synthesized[1]. The first strand cDNA was used as a template for PCR amplification as previously described[1] using the primers HuVH1aBACK and HuVH1FR3FOR. Restriction sites were appended to the repertoires by reamplification using the primers HuVH1BACKSFI and HuVH1FR3FOR. The VH1 repertoires were digested with NcoI and BglII, purified on a 1.5% (w/v) agarose gel, electroeluted, precipitated with ethanol and ligated into the vector pHEN-1-ΨVHB2 digested with NcoI and BglII. The ligation mix was used to transform electrocompetent *E. coli* TG1. Cells were grown for 1 hour in 1 ml of SOC and then plated on TYE medium with 100 μg/ml ampicillin and 1% (w/v) glucose. Colonies were scraped off the plates into 5 ml of 2 × TY broth containing 100 μg/ml ampicillin, 1% (w/v) glucose and 15% glycerol.

Selection of reshuffled libraries. To rescue phagemid particles, 50 ml of 2 × TY containing 100 μg/ml ampicillin and 1% (w/v) glucose (2 × TY AMP-GLU) were inoculated with 10^9 bacterial cells from the library glycerol stock, grown with shaking at 37°C to an A_{600} of 0.9 and then 5 ml added to 50 ml of 2 × TY AMP-GLU prewarmed to 37°C. 2×10^{10} plaque forming units of VCS-M13 (Stratagene) were added and the mixture incubated at 37°C without shaking for 1 hour. The mixture was then added to 500 ml of 2 × TY broth containing 100 μg ampicillin/ml and 25 μg kanamycin/ml and grown overnight at 37°C with shaking. Phage particles were purified and concentrated as previously described[1]. Two rounds (reshuffled light chain library) or four rounds (reshuffled heavy chain library) of enrichment for phOx binding phage were performed in phOx-BSA coated immunotubes (Nunc) (10 μg/ml of 14ox/BSA for selection of the reshuffled light chain library and 10 μg/ml of lox/BSA for selection of the reshuffled heavy chain library). After each round of enrichment, *E. coli* TG1 were reinfected with eluted phage and rescued to provide phage for the next round of panning. For soluble scFv expression, eluted phage was used to infect *E. coli* HB2151 (ref. 37).

Initial characterization of binders with new light chains. Soluble scFv was induced[38] from 94 colonies from each round of selection and analyzed for binding to phOx by ELISA[1]. Twelve clones with ELISA signals stronger than αphOx-15 were sequenced[39] revealing 8 unique clones. The relative affinities of these 8 clones were determined by inhibition ELISAs. For inhibition ELISAs[28], microtiter wells were coated overnight with 100 μg/ml phOx-BSA in PBS and blocked for 2 hours at 37°C with 2% milk powder in PBS. Dilutions of scFv previously determined to result in significant reduction of ELISA values after two-fold dilution were mixed with phOx (10^{-3}–10^{-7} M) in the wells and incubated for 1.5 hours at RT. Bound soluble scFv was detected by ELISA[1]. The concentration of phOx resulting in a 50% reduction in ELISA signal (I_{50}) was calculated for each mutant and compared to that obtained for αphOx15 to determine the relative affinity. Relative affinities, but not the I_{50} value (6.0–400 μM), correlated with affinities measured by fluorescence quench (Fig. 1 and Table 1). Affinities and off-rates of the clone with the highest relative affinity (αphOxB2) as well as αphOx-15 were determined as described below.

Initial characterization of binders with new heavy chains. Soluble scFv was induced[38] from 94 colonies from each round of selection and analyzed for binding to phOx by ELISA[1]. The off-rates of soluble scFv from all ninety positive clones from the third and fourth round of selection were determined by BIAcore (see below) and the clones then grouped according to off-rate and BstNI fingerprint[1]. Eight representative clones were sequenced[39] revealing 5 unique clones. Affinities and off-rates of these 5 clones were determined as described below.

Affinity measurements. Two liter cultures of *E. coli* HB2151 harboring the appropriate phagemid were induced[38] and the soluble scFv affinity purified[2] from the supernatant using the C-terminal peptide tag[40]. For affinity determinations, fluorescence quench titration with the hapten 4-γ-amino-butyric acid methylene-2-phenyl-oxazol-5-one (phOx-GABA) was performed as described[28]. The affinity of αphOx-15 was determined[26,11] using a regime of hapten excess as described previously[1]. Data were averaged from 3 runs. For determination of the affinity of αphOxB2 and the 5 mutants from the shuffled heavy chain library, 100 nM scFv (a concentration ten times the preliminary estimate of the dissociation constant) was titrated with hapten and the fluorescence determined 1 min after each addition[26]. Excitation was at 280 nm and emission was monitored at 340 nm. Data were averaged from 3 to 5 runs. k_{off} was measured by real-time biospecific interaction analysis based on surface plasmon resonance (BIAcore, Pharmacia Biosensor AB)[23,24]. Affinity purified scFv proteins were fractionated on a calibrated FPLC Superdex 75 column (Pharmacia) to eliminate aggregates and the monomeric fraction then used for kinetic measurements. In a BIAcore flow cell, 1300 resonance units (RU) of 100 μg/ml phOx modified BSA (14 phOx/BSA) in 10 mM acetate buffer pH 4.0 was coupled to a CM5 sensor chip[42]. In another flow cell, the sensor chip was activated without phOx-BSA as a control. Adsorption and dissociation of αphOx15 (0.4 μM–2.8 μM) and the other scFvs (80 nM–400 nM) in PBS, 0.2 mM EDTA were measured under a constant flow of 6 μl/min. k_{off} was determined for αphOxB2 and the heavy chain shuffled mutants from the dissociation part of the sensorgram and for αphOx15 from the association part of the sensorgram[43] (necessitated by its rapid k_{off}).

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